INTERPRETATION OF THE ABSORPTION AND CIRCULAR DICHROIC SPECTRA OF ORIENTED PURPLE MEMBRANE FILMS

D. D. MUCCIO AND J. Y. CASSIM, *Department of Microbiology and Division of Sensory Biophysics, College of Biological Sciences, The Ohio State University, Columbus, Ohio 43210 U.S.A.*

ABSTRACT The absorption and circular dichroic (CD) spectra of purple membrane films in which the plane of the membranes is oriented perpendicular to the incident beam are compared with the solution spectra. This enables one to relate structural features of the purple membrane to a coordinate system as defined by a normal to the membrane plane and two mutually perpendicular in-plane axes. The film and solution absorption spectra were similar except for a relative depression in the 200—225-nm region of the film spectrum. However, the CD spectra showed significant differences in the visible region, where the biphasic band in the solution spectrum was replaced by a single positive band at 555 nm in the film spectrum and in the far ultraviolet region, where the 208-nm band was deleted from the film spectra of the native and regenerated membranes. Moreover, a small shoulder occurred at 208 nm in the film spectrum of the bleached membrane. The near ultraviolet spectra also showed differences, whereas the 317-nm band remained essentially the same for both spectra. Based on excitonic interpretations of the visible and far ultraviolet spectra the following conclusions were reached: (*a*) a relatively strong in-plane monomeric interaction occurs between the retinyl chromophore and apoprotein; (*b*) the helical axes of the native and regenerated membrane proteins are oriented primarily normal to the membrane plane; and (*c*) the helical axes of the bleached membrane proteins are tilted more in-plane than the axes of the native or regenerated membrane. Additional conclusions were that an interaction occurs between an in-plane magnetic dipole moment of the retinyl chromophore and probably an in-plane electric dipole moment of a nearby aromatic amino acid(s), and that although the membrane is anisotropic with respect to coupling between electric and magnetic moments of the aromatic amino acids, the transition dipole moments of the aromatic amino acids are not preferentially oriented in either direction.

INTRODUCTION

The purple membrane from *Halobacterium halobium* is a specialized membrane component that is responsible for the photoprocesses of the bacterium. These processes involve the photocycling of a retinyl chromophore (1, 2) that establishes an electrochemical gradient due to the preferential pumping of protons out of the cell (3).

The purple membrane consists of a single protein, bacteriorhodopsin, which constitutes about 75% of the membrane dry weight with the remaining portion being lipids (4). In addition, a single retinyl chromophore is covalently bound by means of a protonated Schiff base to the apoprotein (4–6). The bound chromophore can assume either a 13-*cis* or all-*trans* configuration depending on the amount of visible light (7). Linear dichroic studies of oriented films indicate that the transition dipole moment of the *trans* configuration is oriented 19–27°

out of the membrane plane (8–10), while the transition moment of the *cis* configuration is oriented slightly more in the membrane plane (9). In addition, circular dichroic (CD) studies suggest that the chromophores are dissymmetrically arranged with respect to each other (11–14). Calculated CD spectra based on a cyclic-trimeric exciton model seemingly agree with the observed CD spectra (15, 16).

Information concerning the membrane organization has been provided by x-ray diffraction and electron microscopy studies. The isolated membrane patches are shown to be disks of about 5,000 Å in diameter and 40–50 Å in thickness (17). The proteins are arranged as cyclic trimers forming a hexagonal lattice (17–21). Furthermore, it was shown that the proteins contain seven helical segments of about 30–40 Å in length that are positioned with the helical axes nearly perpendicular to the membrane plane. Estimates of helical content vary from 45 to 80% depending on the method employed (11, 18–22).

Spectral studies of the purple membrane in solution have previously been used to detect structural changes that occur in processes such as light to dark adaptation and bleaching and regeneration (11, 23). However, a solution spectrum can be considered as an average of spectra arising from the orientation of the sample in three orthogonal directions. Often the spectral contours obtained for a particular orientation differ dramatically from that of solution. Therefore, the spectra of oriented samples may contain structural information not available from solution studies. This is especially true for highly anisotropic systems such as the purple membrane that have oriented helical and chromophoric positions.

In this study the absorption and CD spectra of purple membrane in solution and oriented as a film with the membrane planes perpendicular to the incident beam were critically compared over a wide spectral range, 185–800 nm. Interpretation of the results based on a molecular exciton model for the visible spectra (retinyl electronic coupling) and far ultraviolet spectra (amide electronic coupling) yielded structural information that supplements previous findings. Important new findings were (*a*) a relatively strong extrinsic optical activity of the retinyl chromophore in the plane of the membrane, (*b*) a magnetic transition dipole moment of the chromophore oriented in the plane of the membrane, and (*c*) the average tilt of the helical axes of the bleached membrane proteins more in-plane than the average tilt of the helical axes of either the native or regenerated membrane proteins.

MATERIALS AND METHODS

Purple membrane was isolated from *Halobacterium halobium* R_1 as previously described in detail (24). The bleached membrane was obtained by illuminating a suspension of purple membrane with 0.3 M hydroxylamine (pH 7.0) with light from a 500-W DAK projector lamp (D. A. Kadon, Co., Inc., Mount Vernon, N.Y.) filtered through a $CuSO_4$ solution and a number 12 (yellow) Wratten filter (Eastman Kodak Co., Rochester, N.Y.). This was followed by repeated washings with distilled water and centrifugations as previously described (23). Regeneration of the bleached membrane was obtained by microliter additions of all-*trans* retinal in ethanol (10^{-3} M) to the bleached membrane (23). Absorption and CD spectra of the purple membrane preparations were recorded with a Cary 118 C and Cary 60 with 6003 CD attachment (Varian Associates, Instrument Division, Palo Alto, Calif.), respectively, as previously described (11.).

Oriented films were prepared by placing several drops (5–15) of either native or bleached, or regenerated membrane solution (A_{560} = 0.5–2.0, pH 6.6) on the entire surface of a standard cylindrical (22-mm Diam.) or rectangular (1-cm pathlength) quartz optical cell and allowed to air dry slowly in a desiccator at room temperature. The membrane solution was previously degassed and filtered. The same

preparations were used for both absorption and CD measurements. X-ray diffraction, electron microscopy, and linear dichroic studies using essentially this method have indicated that the membrane planes orient parallel to the cell surface upon drying (8, 10, 17–19).

Film nonuniformities are potentially a major source of optical artifacts in both absorption and CD spectra. Optical activity due to form birefringence of an anisotropic film can occur for oriented systems that are not aligned along an optical axis (25, 26). However, linear dichroism of oriented samples prepared in this manner was not detected for the surface of the cells oriented perpendicular to the light beam (8, 10). Furthermore, neutron diffraction results showed minimal mosaic spread from nonuniform stacking of the membrane planes (8). The following criteria were established to judge the spectroscopic quality of the films: (*a*) light scattering in optically transparent regions (such as at 800 nm) of the films should not be greater than that of solution spectra when the solution and film show similar absorbances at 560 nm; (*b*) the CD spectra should not be changed when the cell is rotated about an axis defined by the incident beam or rotated by 180° about any axis that is perpendicular to the incident beam (mirror image); and (*c*) a linear relationship should exist between the absorbances and ellipticities for films of varying thicknesses. We find that only films with absorbances at 560 nm less than 0.1 satisfy all of the above conditions.

Recently, x-ray diffraction studies with 7 Å resolution have indicated no significant structural differences between wet and dry membrane films (21). However, studies have shown that the rates of formation of the intermediates in the photocycle and light- to dark-adaptation of dried films are dependent on the relative humidity of the film (27). Complete dark- to light-adaptation occurs only for relative humidities above 75%. Furthermore, the light-adapted state for films does not occur at humidities below 50%. The humidity of the films in this study was determined by prevailing room condition humidities that varied from 40 to 68%. To insure a constant chromophoric state in the membrane, film studies involved only the dark-adapted membranes.

RESULTS AND DISCUSSION

Visible Spectra

The current interpretation of the visible absorption and CD spectra of the purple membrane is based on a cyclic-trimeric exciton model (15, 16), which considers electronic dipole-dipole interaction between the $\pi - \pi^*$ (NV_1) transitions of only three closely lying chromophores related by a C_3 axis of symmetry (interaction strength depends on the inverse cube of the distance between chromophores). This model predicts that the solution absorption spectrum is composed of three mutually orthogonal transitions, two of which are polarized in-plane (doubly degenerate) and the other is polarized out-of-plane (nondegenerate). The wavelength positions and intensities of the two bands arising from the three transitions depend specifically on the interchromophoric distance and angular orientation of the transition moments of the chromophores (15, 16). Furthermore, associated with each transition is an equal and opposite rotatory strength that results from coupling among the dissymmetrically arranged chromophores about the C_3 axis of symmetry. The sign of the CD bands depends on the sense of the screw axis present between chromophores (28). However, the CD spectra not only arise from excitonic interaction between the dissymmetrically arranged chromophores, but also from the dissymmetric interactions between the chromophore and apoprotein (11–14).

The oriented film absorption spectrum for light incident normal to the cell surface is compared to the solution spectrum of the dark-adapted membrane in the visible region in Fig. 1 *a*. The absorbance of the two spectra have been normalized at 560 nm. The band at 560 nm has been assigned to the $\pi - \pi^*$ (NV_1) transitions of the retinyl chromophore bound to the apoprotein (11). The spectra are identical in the long wavelength region, but deviate in the

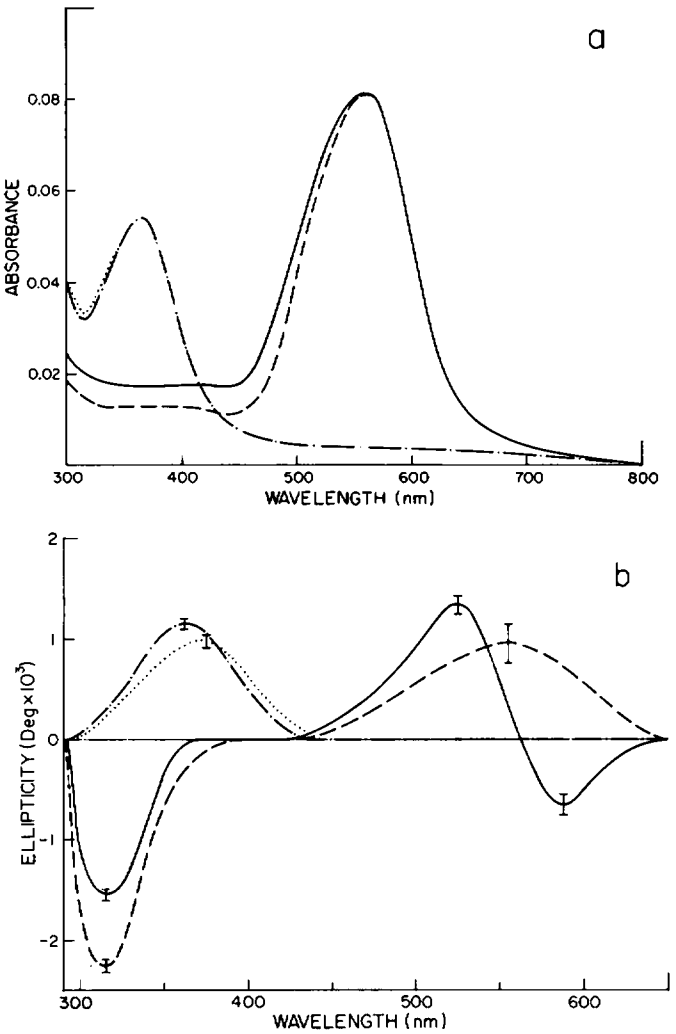

FIGURE 1 Visible absorption (*a*) and CD (*b*) spectra for the native membrane in solution (—) and oriented as a film (----), and for the bleached membrane in solution (- - -) and oriented as a film (· · ·). The solution was measured at a concentration that was a factor 10 times greater than that used in the film spectra. The absorbancies of the spectra were normalized at 560 and 364 nm for the native and bleached membrane, respectively, because the solution spectra conforms to Beer's law in this concentration range. The optical pathlength was 1 cm for the solution spectra.

wavelength region from 300 to 500 nm. This deviation may be due to a relative decrease of light-scattering artifacts in the films or a loss of absorbance due to other $\pi - \pi^*$ transitions of the chromophore that are polarized out of the membrane plane. The wavelength maximum of the dark-adapted oriented film was at 560 ± 1 nm, which is in accord with the maximum of 559 nm usually observed in solution. The band shapes of the two spectra are similar, with the full band width at half maximum absorbance of the film spectrum being slightly less than that of the solution spectrum.

The absorption spectrum of an ideal film oriented with the membrane planes normal to the

incident light should contain absorption bands that are polarized only in the membrane plane. The fact that the wavelength position of the oriented film is similar to the position of the solution spectrum indicates that the in-plane (doubly degenerate) excitonic transitions are almost completely allowed at the expense of the out-of-plane (nondegenerate) excitonic transition. The ratio of the intensities of the two excitonic bands is dependent only on the angular orientation of the electric dipole transition moment of the chromophore out of the membrane plane (15, 16). Linear dichroic results indicate that the transition dipole moment is oriented about 19° out of the membrane plane (8). Thus, according to the excitonic formalism for cyclic–trimeric geometry, the ratio of the intensity of the two excitonic bands must be about 9:1 and independent of the interchromophoric distances.

The wavelength position of the out-of-plane excitonic band can be estimated by curve fitting a band of similar shape as the oriented spectrum so that the sum of the two bands "best fits" the solution spectrum. Optimal position of this band is at 555 nm based on a 9:1 ratio of intensities for the in-plane to out-of-plane bands. Computations of the absorption and CD spectra using curve-fitting procedures based on a cyclic-trimeric exciton model have suggested a much larger splitting between the excitonic bands than the 5 nm estimated from present results (16). However, this lower estimate is in better accord with recent neutron diffraction results (29) and linear dichroic studies (8) that suggest a smaller band splitting.

The fact that the dark-adapted state is an equal molar ratio of *trans* and *cis* configurations of the chromophores (7, 30) does not alter the above argument. According to recent linear dichroic studies (9), a small change in dipole orientation results from the light- to dark-adaptation, which should not change excitonic interaction significantly. In addition, calculations of the absorption and CD spectra for trimer clusters containing either *cis* or *trans* or combinations of these configurations indicate that similar band splittings must occur for all possible chromophoric combinations in the cluster to account for experimental results in the light- to dark-adaptation process.[1]

The visible CD spectrum of the dark-adapted film oriented in a manner similar to that used to obtain the absorption spectrum is compared with the spectrum of a dark-adapted sample in solution in Fig. 1 *b*. The absorbances of the film and solution have been normalized at 560 nm. Instead of a biphasic CD band centered at 563 nm, one positive band occurs in the oriented spectrum with a maximum at 555 nm. The negative band in the solution spectrum at 317 nm occurs in the oriented spectrum as well, but is slightly blue-shifted by 2 nm.

A direct comparison of the intensities of the film and solution spectra is not possible because the chromophores are oriented in the film but are random in solution. However, a comparison can be made based on the assumptions that the membrane planes are oriented parallel to the cell surface and that the solution spectrum is composed of two excitonic bands with a 9:1 ratio of intensities between the in-plane to out-of-plane polarized transitions. The 560-nm absorbance of the film spectrum can be normalized to the absorbance of the in-plane transition to correct for concentration and pathlength differences. Then one can correct the absorbance of the film spectrum by a factor of two-thirds to account for the preferential orientation of the film relative to the solution. The intensity of the positive band at 555 nm in the CD spectrum of the oriented membrane is about one-half the intensity of the positive lobe at 525 nm in the

[1]Muccio, D. D., and J. Y. Cassim. Work in preparation.

solution CD spectrum after this correction. Furthermore, the negative band at 315 nm in the CD spectrum of the oriented membrane becomes essentially equal in intensity to the extremum at 317 nm in the solution spectrum.

The rotatory strength of each excitonic transition, A, for light incident along the Z direction can be expressed as (25, 33):

$$(R_{ZZ})_A = \frac{3e}{2mc} Im\,[(\mu_y)_{OA}\,(\mathrm{ZP_x})_{AO} - (\mu_x)_{OA}\,(ZP_y)_{AO}],$$

where e and m are the electronic charge and mass, respectively, c is the speed of light, $(\mu_x)_{OA}$ and $(\mu_y)_{OA}$ are the matrix elements for the x and y components of the transition dipole moment for the O to A excitonic transition, respectively, and $(ZP_x)_{AO}$ and $(ZP_y)_{AO}$ are the (ZP_x) and (ZP_y) matrix elements for the $(\bar{R}\bar{P})$ tensor for this transition. In the case of the oriented purple membrane, the Z direction is assumed to be normal to the membrane (along the C_3 axes) with the x and y directions both in the membrane plane. Because the transition dipole moment of the nondegenerate excitonic transition is polarized solely normal to the plane of the membrane, the $(\mu_x)_{OA}$ and $(\mu_y)_{OA}$ matrix elements must be identically zero. Thus, this excitonic transition cannot have any rotatory strength. For a given orientation the total rotatory strength of all excitonic transitions must be zero according to the sum rule (25). Therefore, the rotatory strength for the two remaining excitonic transitions must also sum to zero. Because these transitions are strictly degenerate due to the C_3 symmetry of the trimer, no observable CD is possible.

An additional explanation for the loss of the visible CD spectra of the oriented membrane is obtained by rewriting the general expression given in the previous paragraph in terms of the monomeric components. It can be shown that $(R_{ZZ})_A$ can be given by:

$$(R_{ZZ})_A = \frac{3\pi\upsilon_A}{c} \sum_{i=1}^{3} \sum_{j>i} C_{iA} C_{jA} (Z_j - Z_i)[(\mu_y)_i\,(\mu_x)_j - (\mu_y)_j(\mu_x)_i],$$

where υ_A is the frequency for this transition, c is the speed of light, C_{iA} are the coefficients of the excitonic wavefunctions as previously determined for the cyclic trimeric model (15, 16), Z_j is the component of the position vector in a direction that is normal to the membrane plane for the j^{th} chromophore, and $(\mu_j)_i$ is the j^{th} component of the electric dipole transition moment for the i^{th} chromophore in the cyclic trimer. Because the light is incident along the C_3 axis of symmetry, the Z_j components are identical for each chromophore. Therefore, $(R_{ZZ})_A$ is obviously zero for both the nondegenerate and degenerate excitonic transitions.

In view of the results discussed above, the observed CD spectrum of the oriented membrane film should only contain bands due to the dissymmetric interactions between the apoprotein and the chromophore, and not from the excitonic interactions. The CD spectrum of the oriented agrees qualitatively with these predictions. After correcting the absorbance to eliminate the change in intensity due to preferential orientation of the chromophore, only a single positive elliptical band with a Gaussian shape centered near the absorption maximum of the film is evident.

Generally, the interpretation of the CD spectra for oriented molecules can be more complicated than the spectra of solution samples. This arises from the contribution of quadrupole terms to the spectra of the oriented molecule (25, 31–33), which average to zero in

the solution spectra. However, the quadrupole contributions to the visible CD spectrum for a cyclic-trimeric exciton system can be shown to be identically zero for light incident normal to the membrane plane.[2] Thus, the interpretation of the oriented CD spectrum of the purple membrane should not be complicated by these effects.

The 317-nm CD band has previously been suggested to arise from dissymmetric interactions between the chromophore and apoprotein (11). Because no large absorbance is associated with the 317-nm band, the mechanism(s) for induced optical activity must be of the magnetic type (28). One possibility is that the electric quadrupole moment from the magnetically allowed dipole transition of the chromophore could couple with an electrically allowed dipole transition from a neighboring chromophoric group. Recent publications have suggested that an aromatic amino acid(s) is positioned near the protein binding site of the retinal (34) and changes position in the photochemical cycle (35, 36). Therefore, the conditions necessary for magnetic-electric dipole coupling are present in the membrane. The fact that the spectrum of the oriented sample (after normalization so as to correct for the preferential orientation of the chromophore) has approximately the same intensity as the solution spectrum at 315 nm indicates that both the magnetic dipole moment of the chromophore and the electric dipole moment of the aromatic amino acid must be oriented almost totally in the plane of the membrane.

The absorption spectrum of the bleached oriented film is compared to the bleached solution spectrum in Fig. 1 *a*. The absorbances have been normalized at 364 nm. The absorption is due primarily to the retinaloxime, which is most likely bound noncovalently to the protein (23, 37). Because the absorption does not vanish upon orientation, the retinaloxime must have an electric dipole component polarized in the membrane plane. The retinaloxime is bound to the membrane in a manner such that induced optical activity can be observed in the solution spectrum (23). The CD spectra of the bleached membrane oriented as a film and in solution are shown in Fig. 1 *b*. A positive band occurs in the film spectrum similar to that of the solution spectrum of the bleached membrane. However, the wavelength is at about 375 nm, which is red-shifted by 10 nm from the band position in the solution spectrum. The ellipticity of each band is of about the same order of magnitude. Because the position of the retinaloxime is not known, a model cannot be used to directly compare the ellipticities of the two spectra. However, the results indicate that the position of retinaloxime is not preferentially oriented solely along either the in-plane or out-of-plane directions in the membrane system.

Near Ultraviolet Spectra

The near UV absorption spectra of both the native and bleached oriented films are shown in Fig. 2 *a*. The overall profile of the oriented spectrum is not different than those for the solution spectrum. Peaks are evident at 290 and 280 nm with a shoulder at 278 nm in each spectra. However, the intensity of the oriented spectrum for the native membrane is less than that of solution spectrum after normalization at 560 nm. The ratio of the absorbances at 280–560 nm is about 1.2 for the oriented spectrum, in contrast to 1.7 for the dark-adapted purple membrane in solution. However, after correcting the visible spectrum of the film for

[2]Muccio, D. D., and J. Y. Cassim. Unpublished results.

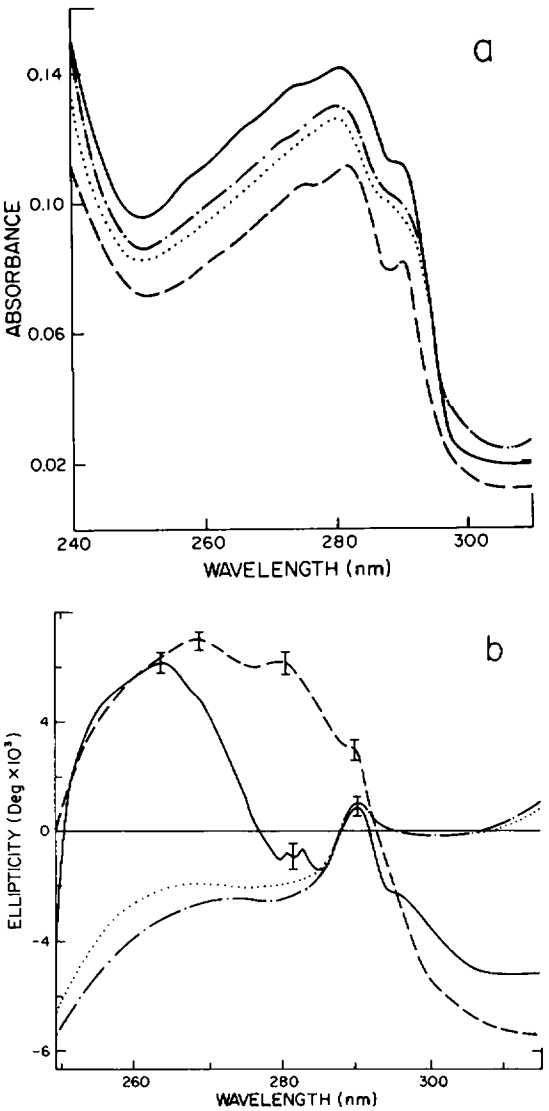

FIGURE 2 Near ultraviolet absorption (*a*) and CD (*b*) spectra for the native membrane in solution (—) and oriented as a film (----), and for the bleached membrane in solution (- - -) and oriented as a film (· · ·). The absorbancies of the spectra were normalized at 560 and 364 nm for the native and bleached membrane, respectively, as indicated in Fig. 1. The optical pathlength was 1 cm for the solution spectra.

preferential orientation, the ratio becomes 1.7. This seems to indicate that the dipole moments of the aromatic amino acids are not preferentially oriented in the membrane system.

The near UV CD spectra of the native and bleached films are shown in Fig. 2 *b*. The spectra of the bleached film and bleached membrane solution are nearly identical. In contrast to the absorption spectrum, however, there are major differences between the film and solution CD spectra of the native membrane. Positive extrema are evident at 290 and 265 nm in the solution spectrum with negative extrema in between. The film spectrum of the native

membrane consists of a shoulder at 290 and positive extrema at 267 and 281 nm, which is very similar to the previously published solution spectrum of L-tryptophan (28). This implies that the optical activity due to the in-plane electric and magnetic dipole moments of the native membrane differ from that of the out-of-plane moments; and, in particular, that the transitions of the aromatic rings of some of the seven tryptophans present in the membrane (34) must lie nearly in the plane of the membrane. However, this is not the case for the bleached membrane, where the band shapes are essentially the same for film and solution spectra. One interpretation of these results is that the native membrane is anisotropic in structure with respect to the in- and out-of-plane direction and that loss of the covalent interactions of the chromophore during the bleaching process eliminates this structural anisotropism.

Far Ultraviolet Spectra

The far UV absorption and CD spectra of α-helical polypeptides can be resolved into three Gaussian bands with extrema at 189, 206, and 222 nm and 190, 206, and 222 nm, respectively (38, 39). The 190-nm band in the CD spectrum is positive, while both the 206- and 222-nm bands are negative. The 222-nm band has been assigned primarily to the $n - \pi^*$ transition of the amide. The $n - \pi^*$ transition is electric dipole forbidden but magnetic dipole allowed based on the local C_{2v} symmetry of the carbonyl group of the amide (40). However, calculations of the molecular orbitals of the amide transitions in a helical geometry have indicated that the enhanced absorbance and induced optical activity observed in the α-helical polypeptide spectra at 222 nm can be accounted for by mixing of the $n - \pi^*$ transition with dipole-allowed $\pi - \pi^*$ transitions (40–42). The 190- and 206-nm bands on the other hand have been interpreted most successfully in terms of a molecular exciton model (41–49). Initially, Moffitt showed that two excitonic bands should result from the Davydov splitting of the amide $\pi - \pi^*$ (NV_1) transition arranged with α-helical symmetry due to strong coupling of these transitions (43). This could result in a doubly degenerate band at higher energies and a nondegenerate band at lower energies in the absorption spectra of α-helical polypeptides with polarizations perpendicular and parallel to the helical axes, respectively. In addition, a positive CD band is associated with the perpendicularly polarized band at higher energies while a negative CD band of equal intensity is associated with the other excitonic absorption band. However, refinements of the original Moffitt calculations by others have since shown that additional excitonic bands besides the ones predicted by Moffitt should occur in each spectrum (47–49). These additional bands have been termed the helical bands because they arise from the excitonic interaction of the perpendicular component of the amide transition arranged in a helical screw axis (47, 48). Many closely spaced absorption bands that are centered at the perpendicularly polarized Moffitt band add to the absorption spectrum. Furthermore, a biphasic CD band that simulates the derivative of a Gaussian band results from the combined CD bands of these transitions (47). Calculations of the CD spectrum of an α-helical polypeptide indicate that the optimal wavelength positions of the bands are 221 nm for the $n - \pi^*$ band, 206 and 188 nm for the Moffitt bands, and 199 and 181 nm for the helical bands (41, 42).

For light incident along the helical axis, symmetry properties of the α-helix require that only the excitonic bands termed helical bands can be present in the absorption and CD spectra

(45–49). Furthermore, the perpendicularly polarized $n - \pi^*$ should persist in the absorption spectrum. However, there should be a significant reduction in the ellipticity in the CD spectrum due primarily to the lending of an electric dipole moment from the parallel polarized $\pi - \pi^*$ transitions to the $n - \pi^*$ transition (42). On the other hand, for light incident perpendicular to the helical axis, the Moffitt bands in addition to the $n - \pi^*$ bands should occur in both the absorption and CD spectra (41, 43). However, additional effects due to the quadrupole terms have been shown to occur in the oriented CD spectrum, which average to zero in the solution spectrum (25, 26, 31–33). For oriented α-helices these terms have been shown to be of equal magnitude to the magnetic terms but primarily affect only the 190-nm CD band (33). The observed spectral bands of oriented polypeptides and proteins have been shown to agree qualitatively with theoretical predictions (38, 39, 50–56).

The far UV absorption spectrum of the oriented native membrane is compared to the solution spectrum in Fig. 3 *a*. The absorbances of the oriented and solution spectra are normalized at 193 nm, because the relatively strong far UV absorbance and relatively weak visible absorption prevent accurate normalization at the same wavelength used for visible and near UV spectra. The absorption spectra of the film and solution are essentially similar. A maximum occurs at 192.5 nm and a shoulder at about 225 nm. However, a depression is observed in the vicinity of 208-nm region of the oriented film absorption spectrum, which is not observed in the solution spectrum. This depression in the oriented spectrum can be attributed to the elimination of the parallel polarized Moffitt band situated in this spectral region. The film absorption spectra of the oriented bleached and regenerated membrane are identical to the film spectra of the native membrane, as has been previously shown for solution spectra (23). Thus, similar conclusions can be reached for the orientation of these membranes.

The far UV CD spectra for the oriented native, bleached, and regenerated membranes are compared to the solution spectrum of the native membrane in Fig. 3 *b*. The CD spectra of the native, bleached, and regenerated membranes in solution have previously been shown to be identical (23). It is apparent, however, that there are significant differences between the solution and film spectra. The solution spectrum is composed of three extrema at 222, 208, and 193 nm with a crossover point at 205 nm. In the film spectrum of the native membrane the 208-nm extremum is completely deleted and there is a reduction in the 222-nm band such that the crossover point is now at about 217 nm. The wavelength positions of the 222- and 193-nm band have been shifted to 225 and 197 nm, respectively. The ratio of intensities of the 197–225-nm band is about 5 for the film spectrum, whereas the ratio of intensities of the 193–222-nm bands for the solution spectrum is about 2.4. The fact that the 208-nm band is completely deleted and a positive band occurs at 197 nm suggests almost complete orientation of the helical axes of the protein perpendicular to the membrane plane according to the excitonic interpretation of the spectra. In addition, the significant reduction of the 222-nm band suggests that a large component of the induced optical activity of the 222-nm band arises from mixing of $n - \pi^*$ transition with the parallel component of the $\pi - \pi^*$ transitions. It should be noted, however, that complexities due to quadrupole moment contributions and changes in effective electromagnetic field due to indices of refraction prevent quantitative correlation of the solution and film spectra.

The depression of the 208-nm band has been associated with light-scattering artifacts in the

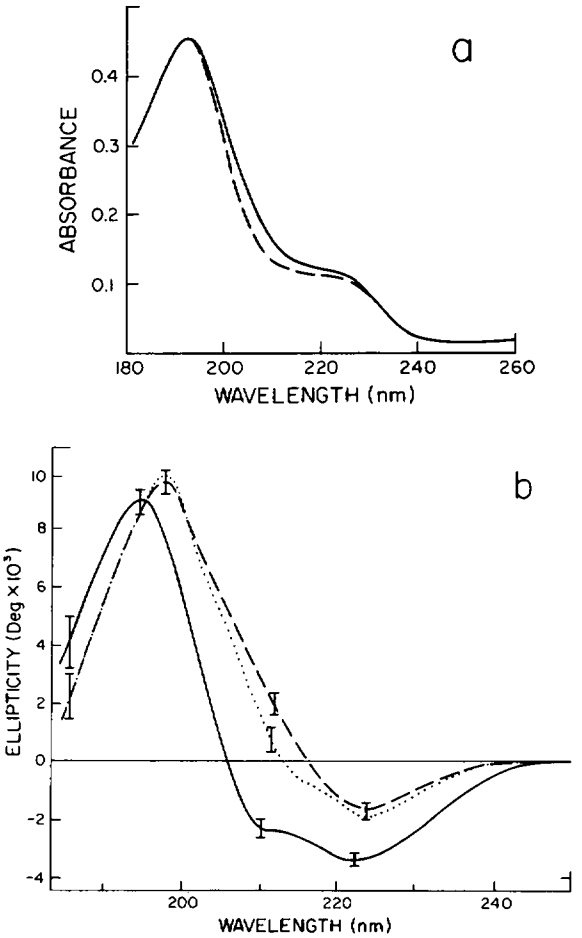

FIGURE 3 Far ultraviolet absorption (*a*) spectra for the native and bleached membrane in solution (—) and oriented as a film (----). Far ultraviolet CD spectra (*b*) for the native membrane in solution (—) and oriented as a film (----), and the bleached membrane oriented as a film (· · ·). The spectra of the bleached and regenerated in solution were the same as the native spectra. The CD spectrum of the regenerated membrane oriented as a film was the same as the native membrane oriented as a film. The absorbancies of the solution spectra were normalized to that of the film spectra at 193 nm. The optical pathlength was 1 mm for the solution spectra.

spectra of membrane solutions in the past (57, 58). However, similarity of the absorbances in a transparent region (such as 800 nm) for both spectra is an excellent indication that the film does not scatter any more light than the solution. In addition, CD studies on highly aggregated membrane samples (aggregated with salt) indicate that the 208-nm band is only depressed due to differential light scattering and absorption flattening, but is never deleted. Thus, the deletion of this band can be attributed most logically to the orientation of the membrane proteins and not to experimental artifacts.

The film CD spectra of oriented bleached and regenerated membrane are also compared in Fig. 3 *b*. A significant finding is that a shoulder is apparent in the film spectrum of the

bleached membrane but is deleted when an identical bleached sample is regenerated with all-*trans* retinal (~90%) and then oriented as a film. Because no spectral change occurs in this region for either native, bleached, or regenerated membrane in solution (23), one can only attribute this change in the film spectra to a change in the average tilt of the helical axes of the bleached membrane proteins further in-plane than either of the helical axes of the native or regenerate membrane proteins. A previous publication has suggested that the hexagonal lattice is distorted in the purple membrane upon bleaching but regains the lattice upon regeneration (59). These spectral results suggest that the distortion is accompanied by a change in the positions of the helical axes of the proteins that are stabilized by the retinyl chromophore.

In conclusion, this study provides evidence that supplements previous findings on the purple membrane structure. The results are in accord with an excitonic interpretation for the visible and far ultraviolet spectra. An important new finding is that the positions of the helical axes are different for the bleached membrane proteins than either the native or regenerated membrane proteins. Furthermore, the results add new and more conclusive experimental evidence for the support of the excitonic interpretations of the spectra of α-helical systems.

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